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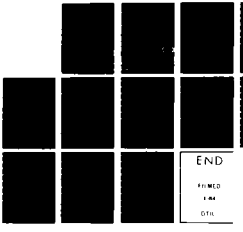

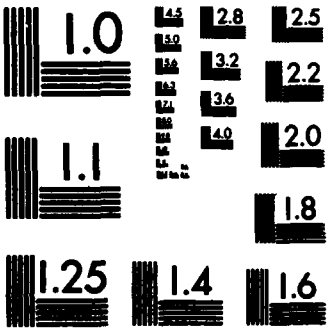

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BLOOD PRESERVATION STUDY

FINAL COMPREHENSIVE REPORT

Ernest Beutler, M.D.

October 1983

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This broad-ranging program has identified various factors involved in regulation of red cell 2,3-DPG and ATP levels during storage. Various storage media have been devised and investigated for their ability to maintain 2,3-DPG, ATP, and post-infusion viability of erythrocytes.

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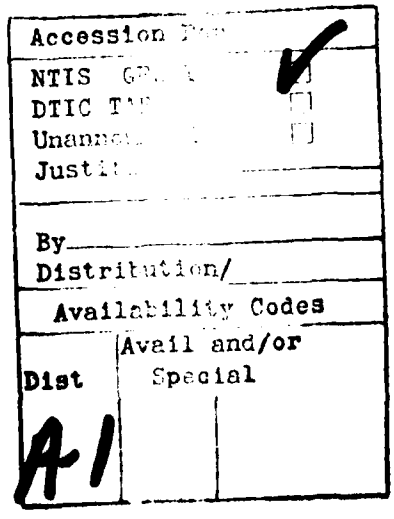

ACCOMPLISHMENTS

A series of studies were carried out in order to determine the rate of depletion of 2,3-diphosphoglycerate from stored red cells, to determine the effect of different storage conditions on the rate of depletion, and to determine the rate of regeneration of 2,3-DPG after reinfusion of stored erythrocytes.

In order to carry out these investigations it was necessary to develop a reliable method for 2,3-DPG estimation which could be carried out on microliter quantities of red cells. For this purpose modifications have been made of the method of Krimsky. A water lysate of erythrocytes is made, the sodium chloride concentration adjusted to 1% by the addition of an equal volume of 2% sodium chloride solution, and deproteinization is achieved by boiling. The effect of the filtrate on the phosphoglyceromutase reaction was then measured by observing the rate of disappearance of phosphoenolpyruvate in a system containing an excess of exogenous enolase. The method of measuring the pH of blood at 4°C was improved by the construction of a new electrode which provides increased surface contact between the glass electrode and the sample. Since red cell 2,3-DPG levels are greatly dependent upon pH, the development of this new instrument is proving very useful in evaluating blood storage systems. Using this technique the effect of pH of the preservative medium and of the presence of adenine and phosphate on the rate of loss of 2,3-DPG from stored blood was estimated. It was shown that 2,3-DPG

disappearance is strongly pH dependent, and that better preservation of 2,3-DPG is obtained when higher pH levels are employed in the preservative. However, the disappearance of ATP in the early phases of storage was found to be more rapid when high pH levels are employed. Even slightly higher pH levels than those encountered with ACD blood were helpful in maintaining 2,3-DPG levels during the first two weeks of storage. Thus, CPD solution was found to be slightly superior to ACD in maintaining 2,3-DPG levels of red cells.

Viability studies were carried out on red cells stored at relatively high initial pH levels. These experiments suggested that the use of high initial storage pH levels does not represent a feasible approach to counteracting the rapid 2,3-DPG loss of stored red cells. In the course of these studies it was also found that the addition of adenine slightly, but statistically significantly, hastened the rate of 2,3-DPG depletion of ACD-stored blood. These findings suggest that although the viability of blood stores in ACD-adenine for periods of 35 days of more is vastly superior to that of red cells stored in ACD solution alone, ACD blood may be physiologically superior during the first week of storage.

In order to determine the importance of 2,3-DPG depletion, and the consequent shift of oxygen dissociation curves on patients receiving transfusions of stored blood, studies were carried out to determine the rate at which 2,3-DPG was regenerated in reinfused red cells. The first series of studies were

undertaken under *in vitro* conditions designed to stimulate the conditions encountered by red cells *in vivo* after transfusion. These studies showed that the regeneration of 2,3-DPG was sufficiently slow that even after 8 hours incubation only approximately one-third of the lost 2,3-DPG had been regenerated.

Further studies were carried out in patients with blood group A who required transfusion. The recipient cells were labeled with ^{51}Cr on the day prior to transfusion. The patients were then transfused with blood which had been stored in ACD solution for 17 to 20 days and the transfused cells were separated from recipient cells by a rapid technique of differential agglutination developed for this purpose. Although there was contamination by recipient cells, correction could be carried out by estimating the ^{51}Cr activity of the recovered cells. Such investigations disclosed that the rate of regeneration of 2,3-DPG *in vivo* was considerably greater than that estimated from the *in vitro* studies. Nearly one-half of the 2,3-DPG lost during storage was regenerated within four hours.

Although we had originally planned to study the actual shift in the oxygen association curve of the recovered red cells using the mixing technique of Edwards, study of this technique has disclosed that the amounts of recovered cells required were greater than we could recover by differential agglutination. However, since data of others shows an excellent correlation between the 2,3-DPG levels and the oxygen association curve, it may be presumed that considerable normalization of the oxygen

association curve of transfused erythrocytes had occurred by four hours. These studies suggest that 2,3-DPG levels will be of considerable importance when a large number of blood transfusions are given rapidly, as in massive wounding.

Efforts were also made to develop additional means for estimating the viability of stored red cells. Because N-ethyl maleimide (NEM) was believed to bind irreversibly to red cell glutathione (GSH) without affecting red cell viability, it was thought that ^{14}C NEM might prove to be a useful label. Accordingly, preliminary studies were carried out in rabbits whose red cells had been doubly labeled with NEM and Chromium^{51}. These studies showed that the NEM label was rapidly lost from the red cell. Thus, NEM proves not to be a suitable label for red cell survival studies, but the loss of NEM from red cells is a surprising phenomenon which may be of some biological importance.

Red cells from ACD-collected blood were stored in artificial media containing glucose, adenine, phosphate, and a bicarbonate-carbonate buffer. Excellent results have been obtained after storage of pilot (50 ml) units for 42 days, both with respect to viability and 2,3-DPG levels. It was found that periodic mixing of the units was essential if good 2,3-DPG preservation was to be achieved. One to 3 percent of the red cells hemolyzed during the storage period. The removal of leukocytes and platelets had no effect on the preservation of the red cells.

Scaling-up these investigations to 500 ml units of blood

(200 ml of packed red cells), resulted in markedly impaired 2,3-DPG preservation. Further investigations showed that this was due to a more rapid fall in the pH of red cells stored in large bags than those stored in small bags. Studies with ^{14}C labelled bicarbonate buffer showed that the stabilization of the pH of stored red cells in artificial media depended upon the passage of carbon dioxide through the plastic film. When the surface volume ratio of the storage container fell below a critical value, the rate of fall of pH was too great to permit retention of red cell 2,3-DPG. Furthermore, it was found that plastic materials from some manufacturers (e.g. Fenwall) permitted much better stabilization in pH and 2,3-DPG preservation than plastic materials obtained from another manufacturer (McGaw). An additional problem which was encountered is <u>in vitro</u> hemolysis which is somewhat greater than would be desired.

A bicarbonate-adenine-glucose-phosphate-mannitol (BAGPM) preservative solution was developed. This solution is added to red cells which have been collected in a conventional preservative CPD or ACD solution. Formed elements and plasma are removed, and are immediately available for fractionation. BAGPM is then added to the erythrocytes and they are agitated periodically. A large variety of plastic films were studied to determine their similarity for this system. These include 15 ml polyvinyl chloride (PVC) silastic, PVC coated silastic polyethylene. It was apparent that silicone rubber (silastic) allows CO_2 to be lost very rapidly. However, the rise in pH which occurred resulted in ATP depletion.

Calcium hydroxide, either in small sialastic bags ("baggies") or embedded in sialastic blocks ("blockies"), was investigated as an internal CO_2 absorbing devices.

$Ca(OH)_2$ added to blood produced marked hemolysis. Hence, leakage of calcium hydroxide from the sialastic membrane "baggie" constituted a serious potential hazard. Therefore, the use of calcium hydroxide embedded sialastic blockies of 3 x 3 x 1 cm size were studied as replacements of the old baggies.

Studies were carried out with varying amounts of calcium hydroxide: baggies containing 6 gm, 3 gm and 1 gm calcium hydroxide were tested and it was found that the most satisfactory amount to provide adequate preservation of 2,3-DPG without compromising the levels of ATP, was in the 3-6 gm range. Blockies containing 6 g $Ca(OH)_2$ were placed into a standard blood bag containing ACD A. It was observed initially that calcium hydroxide shed from the raw surfaces when stored at room temperature in ACD and to some extent also in CPD. These difficulties were accentuated after autoclaving in ACD. They were overcome by providing a coating of liquid sialastic over the surface of the "blockie" and then enclosing it in another sialastic membrane. This prevented leaching of calcium hydroxide from the raw surfaces without affecting the CO_2 entrapment function of the blockie. After collection of blood and removal of plasma, BAGPM was added to the packed cells. A "blockie" containing 6 g $Ca(OH)_2$ proved to be a very satisfactory way to

trap carbon dioxide generated from the bicarbonate buffer of the BAGPM medium. Using this system, pH levels were remarkably stable throughout the 42 day period of storage. At the end of storage, 2,3-DPG levels were 92% of the original levels, while approximately 62% of initial ATP levels were maintained. "Blockies" with 4 gm calcium hydroxide were also tested. However, it was noted that when only 4 gms of calcium hydroxide was employed, 2,3-DPG levels were not maintained unless the bags were very frequently mixed. Hence, it appears that 6 gms of calcium hydroxide is the optimum amount (see Appendix IX). It is of interest to note that the blockies were also superior to the baggies in the maintenance of 2,3-DPG and ATP for the full 42 days of storage whether the units were mixed once a week or five times a week.

Microaggregate formation in BAGPM units with baggies or blockies was shown to be markedly diminished. These data were further confirmed by the measurement of screen filtration pressure in all the units studied. Because of the marked diminution in the content of white cells, platelets, and fibrinogen, the interaction of these elements is probably much less in these units and therefore, the screen filtration pressure remains much lower than in conventional blood units stored in ACD, CPD, or CPD II-adenine. Whenever screen filtration pressure rose, it could be returned to normal by filtration through the usual blood filter utilized in the administration of blood to the patients. Therefore, it is anticipated that whatever

microaggregates form, they would be readily cleared by the standard large-pore size blood filters conventionally used in the blood administration to humans.

We found that loss of ATP during storage can largely be prevented by the addition of pyruvate, in quite small quantities, to the storage medium. This effect of pyruvate is apparently exerted through shift in the lactate/pyruvate equilibrium, making available more NAD for the glyceraldehyde-phosphate dehydrogenase step. Preservation of ATP and 2,3-DPG in blood collected in ACD solution and in CPD solution adjusted to various pH levels was investigated.

On the basis of these findings ACD and CPD media with pH levels of 7 or 7.5 were selected for further study. Investigations were carried out using such preservatives with and without the addition of adenine, and with and without the addition of pyruvate. We have found that when the pH of CPD-adenine solution was adjusted to 7.5 normal levels of 2,3-DPG are maintained for 18-21 days. If 1 mM of pyruvate is added to the CPD-adenine solution, ATP levels were superior. Using 7 ml autotransfusions, we found viability to be greater than 70% after 42 days storage.

Ascorbic acid was found to exert a markedly favorable influence on red cell 2,3-DPG levels in such solutions, and the viability of the stored blood was found, in preliminary studies, to be excellent. In efforts to clarify the mechanism of the effect, studies were carried out on the blood of a patient with

virtual absence of NADH diaphorase and on the blood of two G-6-PD deficient subjects. In both of these instances, 2,3-DPG preservation was excellent, and there was no increase in the methemoglobin content of the blood from the patient with NADH diaphorase deficiency. These studies indicate that the ascorbate effect is not mediated either through methemoglobin formation, NADH diaphorase activity, or, through the hexose monophosphate pathway.

Mixing of blood during storage was found to exert a profound effect on the biochemical properties of red cells during storage. We discovered that periodic mixing during storage increases the levels of 2,3-diphosphoglycerate, and probably as a consequence of this, results in lower glucose consumption and lactic acid production of the red cells. In a variety of media, including CPD, CPD-adenine and CPD with various additives such as ascorbic acid and dihydroxyacetone, and BAGPM mixing exerted a 2,3-DPG-sparing effect. The studies of the effect of mixing were extended to packed red cells. Here, it might have been anticipated that mixing would have little effect, since there would be no plasma with which to mix the red cells. Somewhat surprisingly, the same beneficial effect of mixing was observed.

<u>In vitro</u> investigations were performed to determine whether the composition of CPD-adenine was adequate for the preservation of cells packed immediately after collection to 90% hematocrit. A medium which could preserve cells at this hematocrit could obviously increase greatly the availability of plasma components,

many of which are in short supply. It was found that the concentration of adenine could be reduced to 0.25 mM (final concentration in blood-preservative mixture) without impairing the ATP preservation of the erythrocyte. However, in the 90% packed cells, the concentration of glucose normally provided by CPD solution was inadequate for maintenance of ATP levels throughout 35 days of storage.

Since dihydroxyacetone (DHA) seems a very promising additive for 2,3-DPG preservation, basic studies of metabolism of DHA were carried out. These included a characterization of the enzymes responsible for the phosphorylation of DHA by erythrocytes, triokinase. The metabolism of 2,3-DPG by intact erythrocytes was also investigated. It was found that DHA was readily metabolized by intact red cells, and could, under appropriate circumstances, replenish depleted 2,3-DPG levels. The effect of DHA on preservation of 2,3-DPG at 4^{o} storage was also confirmed in this investigation. It was found that ascorbic acid has a remarkable effect upon the maintenance of 2,3-DPG in whole blood, even in the relatively acid preservative medium.

Additional attempts were made to study the mechanism of the ascorbic acid effect on red cell 2,3-DPG levels. In an effort to distinguish between an effect on increased synthesis as opposed to decreased degradation, investigations were carried out in blood stored with ^{14}C glucose, measuring the labeling of the 2,3-DPG pool. They suggest that both increased synthesis and decreased degradation may be involved.

The effect of combined DHA and ascorbic acid on red cell preservation was studied at different pH levels.

This laboratory served as a participant in the collection of viability studies in human volunteers with blood preserved in this new medium, CPD-A1, with a cooperative group under the IND filed with the FDA by Fenwal Laboratories. A total of 18 volunteers who met the usual AABB standards were studied in our laboratory. Of these, 8 were studied with packed cell units and the other 10 with whole blood units. All the units of whole blood stored in CPD-A1 had 24 hour viability of at least 72% at the end of 35 days of storage. However, the preliminary studies with 5 packed cell units showed that 2 of these 5 packed cell units did not have adequate glucose (and hence ATP) at the end of 35 days. Therefore, at the first meeting of the members of cooperative study group, it was mutually agreed to study the subsequent packed cell units at the end of 28 days. At the end of this period, all three remaining packed cell units showed better 75% viability at 24 hours. Subsequently it was discovered that through an administrative error at Fenwal Laboratories PL 130 rather than PL 146 had been employed in preparing the bags containing CPD II. It therefore became necessary to repeat the investigations using authentic PL 146 bags as the primary container. The results were quite similar to those obtained in PL 130.

In both of these series of studies, it was observed that

most or all of the glucose was exhausted from cells in some units packed at hematocrits of 75 ± 5% and stored for 35 days. For this reason, two new preservatives, believed to represent improved formulations for storage of cell concentrates have been devised and manufactured by Fenwal Laboratories. These formulations, designated CPD-A2 and CPD-A3 contain respectively 1.75 and 2.00 times the amount of glucose present in CPD and sufficient adenine to provide a concentration of 0.5 mM in the blood-preservative mixture.

We also evaluated the adequacy of CPD-A1 for storage of packed red cells and feasibility of using CPD-A2 or CPD-A3 as substitute preservatives.

In our studies of this preservative a number of possible predictive parameters for red cell preservation were investigated. These included not only the measurement of ATP, a standard, albeit unreliable parameter of red cell viability, but also by measurement of deformability in the ektacytometer, measurement of red cell phosphofructokinase activity, agglomerability of the red cells according to the method of Meyerstein et al.

Basic studies of the effect of storage of platelets in CPD and CPD-adenine solutions were undertaken. These included measurements of platelet glycolytic enzymes, of platelet metabolic intermediates during platelet storage and after incubation under physiologic conditions at storage, and studies of capacity of platelets to utilize citrate. In order to

determine whether increased glucose concentration adversely affected the storageability of platelet concentrates by hastening the fall of pH, platelet concentrates were prepared in CPD, CPD-A1, and CPD-A3 in order to provide a maximum range of glucose and adenine concentrations for study. The rate of glucose consumption, lactate formation and the net accumulation of hydrogen ions was compared not only to the platelet count of the preservative solution but also to the residual white cells which were present. These investigations show that CPD-A3 does not have an adverse metabolic effect on platelets. Moveover, they emphasized the important role that residual white cells may play in the storage of platelet concentrates. It is likely that the earlier results suggesting that high glucose concentrations might adversely affect platelets may have been artifactual due to increased residual numbers of leukocytes.

Four hundred and fifty milliliters of blood were collected from each of 10 volunteer donors. After an 8 hour holding period at room temperature the red cells were packed to a hematocrit of 80% and stored at 4°C, 2 units for 42 and 8 units for 29 d without agitation. Storage position of the blood was alternated between lying and standing. Viability studies were carried out on all 10 units. Blood stored in the lying position did not differ from blood stored in the standing position for 42 d, no significant difference has been observed in the studies, carried out at 49 d. The viability of these samples was 69.09% ± 5.2% ($\bar{x}$ S.D.). There was no significant correlation between 24 hour

viability, on the one hand, a post-storage ATP levels on the other ($r = 0.07 \pm 0.41$). An undesirable degree of hemolysis was also present, plasma hemoglobin levels ranging between 0.78 and 2.70 g/dl. Moreover the amount of hemolysis was significantly negatively correlated to viability ($r = 0.69 \pm 0.21$).

In the course of these investigations various auxiliary studies were undertaken to aid in better definition of the "storage lesion". The osmotic fragility of stored red cells was investigated both before and after reinfusion into volunteer donors. The osmotic fragility of red cells stored in CPD-A2 was found to be greatly increased when measured directly by estimating lysis in graded phosphate-buffered salt solutions. Much of this increase in osmotic fragility was found to be due, however, to the accumulation of lactate in the stored cells. Lactate leaves red cells only slowly and therefore exerts a marked osmotic effect. When stored red cells were pre-equilibrated with isotonic sodium chloride solutions until the lactate had been removed from the cells the osmotic fragility was increased to a much smaller extent. However, a "tail" of osmotically fragile cells could still be detected. Studies to determine the fate of these cells were performed by devising a method of sequential hemolysis which permitted us to measure the osmotic fragility of the reinfused cells using the release of ^{51}Cr from the labeled cells as an indicator of hemolysis. These investigations demonstrated that the osmotic fragility of stored cells rapidly returns to normal after their reinfusion into the circulation. The normalization of the osmotic fragility of reinfused

cells does not apparently depend principally upon removal of the most fragile cells, but rather on correction of the increased osmotic fragility of cells in the circulation. This correction is due to regeneration of 2,3-DPG after reinfusion.

We have also compared the size of the fragile tail with in vivo viability. These studies showed little correlation between these measurements.

The most commonly used method for the evaluation of the viability of stored red cells is the "single isotope" technique in which a red cell mass estimation is made by back-extrapolation of ^{51}Cr radioactivity after infusion of the stored, labeled erythrocytes. This technique has been criticized because of the possibility that a bias may be introduced by loss of non-viable erythrocytes in the first 5 minutes after infusion, before the first sample is taken. We evaluated the magnitude of this bias by performing simultaneous red cell mass estimations with fresh red cells from the same volunteer using ^{99m}Tc as a label and comparing this "true" red cell mass with the one obtained by back-extrapolating the ^{51}Cr activity. These investigations showed that the ^{51}Cr did produce a small and quite reproducible overestimation of the viability of red cells, averaging about 3% over a wide range of values. These investigations also showed that best estimates of red cell mass were obtained by sampling only between 5 and 15 minutes following the infusion of the stored, labeled red cells.

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Personnel	Funding year
Ernest Beutler, M.D. Lee A. Wood, M.D.	1968
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Ernest Beutler, M.D. Carol West	1982 (Revised)
Ernest Beutler, M.D. Carol West Terri Gelbart	1983
Ernest Beutler, M.D. Carol West	1983 (Revised)

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